direction. This data suggests that Vin contributes to the passive mechanical properties of the myocardium, and disrupting the mechanical linkage between the cytoskeleton and the cell membrane reduces the overall stiffness of the myocardium.

2571-Pos Board B541

Quantitative Assay of Skeletal Muscle alpha-actin Expression In Normal and Pathological Human and Mouse Hearts

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We have developed a polyclonal antibody specific to skeletal muscle actin (ACTA1) in the presence of cardiac actin (ACTC) and have used it to quantify the skeletal actin content of human and mouse cardiac muscle. Heart muscle myofibrils were separated by SDS-PAGE and Western blotted. The membrane was stained first with MemCode total protein stain and then probed in with the anti-skeletal muscle actin antibody, visualised by ECL. The ECL band signal was normalised to MemCode-stained actin band. Skeletal muscle myofibrils were used as a 100% skeletal actin standard. For the negative control we used myofibrils from skeletal muscle of a skeletal actin knockout mouse crossed with a transgenic mouse over-expressing cardiac actin in skeletal muscle. There was no detectable signal from the skeletal actin antibody in the pure cardiac actin control.

Human non-failing donor heart muscle contained $21 \pm 2\%$ skeletal actin (n=9). This is comparable to previous estimates using N-terminal sequencing or Mass spectroscopy. In both end-stage failing heart muscle and in myectomy samples from HCM muscle the skeletal actin content was much higher ($58 \pm 5\%$ in both cases, n=12, 11). The increase in skeletal actin content of myopathic muscle was highly significant ($p < 0.001$). Mouse heart muscle (C57BL/6 strain) contains $26 \pm 3\%$ skeletal actin (n=7). This is similar to human heart. ACTC DCM mutation E361G expressed at 50% in mouse heart has $16 \pm 3\%$ (n=8) skeletal actin but ACTC HCM mutation E99K expressed at 50% is not significantly different from NTG $24 \pm 2\%$ (n=5). We conclude that in human heart, acquired heart failure or failure secondary to HCM is associated with an increased content of skeletal muscle actin. In contrast, in mouse genetic models of HCM and DCM skeletal muscle actin content may be lower than normal.

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Structural and Functional Characterization of Cardiac Troponin T Mutations in the TNT1 Domain That Cause Familial Hypertrophic Cardiomyopathy

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FHC is a primary cardiac muscle disorder that is one of the most common causes of sudden death in young people. FHC "hotspot" mutations at residue 92 in cardiac troponin T (cTnT) flank the proposed α-helical TNT1 tail domain whose flexibility has been suggested to be important in normal protein-protein interactions within the thin filament. Through Molecular Dynamics (MD) simulations, we showed that FHC mutations Arg92Leu, Arg92Trp, and Arg92Gln cause local α-helical structural changes and increased flexibility at a critical hinge region 18 Angstroms distant from the mutation. We have extended this MD analysis via the use of a self-defined coordinate to measure localized bends in the helix and found that forces acting on this bending coordinate are lower in mutants than wildtype. This quantitatively suggests a less restrictive bending motion in mutants explaining the increased flexibility of the hinge region. To determine how primary biophysical changes induced by these mutations cause complex cardiomyopathies we hypothesize that flexibility alterations and changes in force within compaction-expansion regions in mutational segments lead to electrostatic perturbations, possibly interfering with cTnT-TM complex formation and thin filament function. *In vitro* motility assays with wildtype cTnT and hotspot FHC-cTnT mutants are in progress to directly correlate predicted alterations in electrostatic properties with resultant functional changes. Moreover, contractile and Ca^{2+} transient measurements on isolated myocytes address downstream myocellular responses to the mutation's primary perturbation on structure and function. Data showed normal percent shortening in Arg92Leu myocytes while Arg92Trp percent shortening was significantly impaired compared to Non-Tg ($4.740 + 1.165$ vs. $6.971 + 2.098$, $p<0.001$). Completion of these studies will directly address the links between thin filament structure/function, downstream myocellular responses, and resultant distinct cardiovascular phenotypes.

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Structural and Functional Characterization of cTnT in Familial Hypertrophic Cardiomyopathy

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Familial Hypertrophic Cardiomyopathy (FHC) is a primary disease of the cardiac sarcomere. Many disease-causing mutations in the thin filament protein cTnT are found within the TNT1 region. Residues 160-163 represent a mutational hotspot within a highly charged region (158-RREEEENRR-166). In this region, this highly alpha helical domain may unwind to create a flexible hinge that is necessary for function, the structure and dynamics of which may be affected by FHC mutations. We are investigating the structure and function of this region using in vitro motility (IVM) assays and SDSL-EPR. The purpose of our IVM experiments is two-fold: to functionally analyze our spin labeled proteins and to gain insight into the function of TNT1 in the presence of cysteine substitutions and FHC mutations. Preliminary IVM data shows a progressive increase in the severity of the functional effects of cysteine substitution and spin labeling across the putative hinge region ($153<168<172$), suggesting that this region is dynamically important and may be making critical interactions with other components of the sarcomere. Preliminary CW-EPR spectra show an increase in isotropic rotational rate at residue 153 (upstream of the putative hinge region) between cTnT alone and in the troponin ternary complex, suggesting that there is a decrease in alpha helical character at this residue in the ternary complex. Introduction of Δ160E further increases the isotropic rotational rate, suggesting an increase in flexibility due to backbone changes induced by the mutation. To further investigate structural and functional changes caused by FHC mutations within the putative hinge region, we will continue to expand our IVM functional analyses with additional cysteine substitutions, as well as FHC mutations at residues 160 and 163. Double label SDSL-EPR are currently underway that will provide secondary and tertiary structural information.

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FHC-linked Mutations in the Myosin Regulatory Light Chain Interfere with RLC Phosphorylation in Transgenic Mice

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Previous studies have shown that MLCK-phosphorylation of the ventricular regulatory light chain (RLC) increases myofilament Ca^{2+} sensitivity, maximal level of force and rate of tension development thereby enhancing the systolic function of the heart. We have investigated the effect of FHC (familial hypertrophic cardiomyopathy) mutations in the myosin RLC on its phosphorylation measured in rapidly frozen ventricular samples from transgenic (Tg) mice. We observe that the Aspartate to Valine substitution in Tg-D166V mice and Arginine to Glutamine in Tg-R58Q mice result in a decreased phosphorylation of RLC detected in left ventricular samples from Tg-mutant compared to Tg-WT mice expressing the human ventricular RLC. The level of RLC phosphorylation was determined by Western blotting utilizing human specific phospho-RLC antibodies (gift from Dr. N. Epstein, NIH). Our data from skinned Tg-D166V and Tg-R58Q papillary muscle fibers show that both RLC mutations lead to a decreased maximal level of force and to slower kinetics of force generating myosin cross-bridges compared to WT fibers. Studies in intact papillary muscle fibers show prolonged force transients for both D166V and R58Q mutants. In addition, the hearts of aging mutant mice demonstrate histopathological changes and frequent occurrences of fibrotic lesions. Clinical studies revealed that both D166V and R58Q mutations are associated with severe FHC phenotypes with multiple cases of sudden cardiac death. Our results suggest that phosphorylation of RLC plays an important role in regulating cardiac function and its deficit may contribute to malignant FHC phenotypes. Reduced RLC phosphorylation observed in the D166V and R58Q hearts correlates with our cellular findings and could be responsible for delayed force transients, slower cross-bridge kinetics and decreased force observed in Tg-D166V and Tg-R58Q papillary muscle fibers. Supported by NIH- HL071778 (D.S-C.).

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Mouse HCM Model Expressing E99K ACTC Mutation Reproduces Phenotypes As Found In Human Patients

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